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Ex Vivo evaluation of the mucolytic effect from a natural herbal combination of *Echinacea purpurea*, *Sambucus nigra*, *Glycyrrhiza glabra*, *Vitex trifolia*, and *Zingiber officinale*.

Rosalina Wisastra*, Poppy Firzani Arifin.

SOHO Centre of Excellence in Herbal Research (SCEHR), SOHO Global Health, Pulogadung 6, Jakarta 13920, Indonesia.

ABSTRACT

Mucus plays an important role in respratory system as moisturizing agent and physical of the innate immune system. The treatment for the overproduction of mucus upon the occurence of allergy or infection has gained considerable attention over the years. This study aimed to evaluate the mucolytic activity of natural herbal combination (NHC) containing *Echinacea purpurea* herba dry extract, *Sambucus nigra* (elderberry) fructus extract, *Glycyrrhiza glabra* (liquorice) radix extract, *Vitex trifolia* (Indian black pepper) folium extract, *Zingiber officinale* (ginger) rhizoma extract by ex vivo. The mucolytic activity of this NHC with various dose concentration 0.25%, 0.5%, 1.0% and 2.0%, was examined by measuring the viscosity of mixture of cow intestinal mucus and phosphate buffer pH 7 using Viscometer Brookfield LVDV II. N-acetylcysteine (NAC) 0.1% w/v was used as the standard mucolytic drug. Viscosity values were analyzed statistically using ANOVA single factor to determine differences between treatment groups. Our finding shows that all NHC group has a mucolytic effect and the mucolytic effect was identified as dose-dependent . The mucolytic effect from 0.5% of NHC is statistically equal to the mucolytic effect from 0.1% NAC. This result could offer a potent plausibe approach for new mucolytic medication. However, further exploration regarding the other efficacy in mucoactive and its possible mechanism of action are prompt to be done in the future.

Keywords: Mucolytic, *E.purpurea, S.nigra, G.glabra, V.trifolia, Z.officinal,* N-Acetyl cysteine

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Corresponding author

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INTRODUCTION

The respirartory airways are lined by a layer of protective mucus gel that located at atop of watery periciliary fluid. Mucus is an adhesive, viscoelastic gel polymer containing inorganic salts, antiseptic enzymes, immunoglobulins, and glycoproteins such as lactoferrin and mucins. The biophysical properties of which are largely determined by entanglements of long polymeric gel-forming mucins, MUC5AC and MUC5B. Mucus is essential for body function and acts as a protective and moisturizing layer to keep critical organs from drying out. This layer protects the airway from inhaled irritants and also entraps and clears bacteria, inhibits bacterial growth and biofilm formation. Volume of mucus produced by oropharyngeal and tracheobronchial mucus glands, at a healthy human is approximately 2 L per day (Elman, 2005).

Overproduction mucus is usually occur during any alteration in respiratory system, allergy or infection are often the main cause that lead to excess mucus production. During the period of infection, the mucus contains the viruses or bacteria responsible for the infection as well as infection-fighting cells of the body's immune system such as white blood cells. Those combination lead the formation of phlegm. High amount of phlegm could obstruct the airflow and ultimately will lead to development of respiratory diseases, such as bronchitis, asthma and Chronic obstructive pulmonary disease (COPD) (Shale, 2004).

Mucoactive medications are medications that are able to reduce mucus/phlegm viscosity, and induce mucus/phlegm excretion (Dhar, 2013). Mucolytic drug is one of mucoactive medication that change the biophysicalproperties of secretions by degrading the mucin polymers, Deoxyribonucleic Acid (DNA), fibrin, or F-actin in airway secretions, and thus decreasing its viscosity (Table 1) (Alam, 2013).

Classic mucolytics mechanism of action is through depolymerizing the mucin glycoprotein oligomers by hydrolyzing the disulfide bonds that link the mucin monomers. Breaking the disulfide bond is usually accomplished by free thiol (sulfhydryl) groups, which hydrolyze disulfide bonds attached to cysteine residues of the protein core (Figure 1). The well known of these agents is NAC. NAC can decrease mucus viscosity *in vitro*, thus could be potent as mucolytic medication (Sheffner et al; 1964).

Another class of mucolytics drugs are the peptide mucolytics, which are designed specifically to depolymerize the DNA polymer (dornase alfa) or the F-actin network (eg, gelsolin, thymosin β4) and are most effective when mucus/phlegm is rich in DNA pus. Aerosolized dornase alfa able to reduce the viscosity and adhesiveness of infected mucus *in vitro* (Shak et al., 1990). Under proper conditions, actin which is the most prevalent cellular protein in the body, polymerizes to form F-actin. F-actin plays a vital role in maintaining the structural integrity of cells. Extracellular F-actin probably contributes to the viscoelasticity of mucus, however this role of F-actin still unclear and has not been definitively investigate (Tomkiewicz et al; 1996) (Puchelle et al; 1998).

Beside from in the respiratory organs such as nose, throat, lungs, and sinuses, mucus is also produced in another site in the body including the gastrointestinal tract. Mucus in the respiratory tract contains 97-98% of liquid components and 2-3% of solid components including protein, mucin, inorganic salt, lipid (Jacquot et al; 1992)(Fahy et al; 2010). Mucus in the gastrointestinal tract contains 98% of liquid component and 2% of solid components (Pelaseyed et al; 2014). Since both mucus has a similar composition, mucus from gastrointestinal tract are often to use as an ex-vivo model for respiratory mucolytic study. Some expectorant drugs had been tested for its mucolytic effect using the ex-vivo model mucus from swine stomach (Misawa and Imamura, 1988).

Although different approach of mucolytic medication has been investigated for decades, only a few mucolytic agent from natural herbal has been defined. Several type of natural herbal are claimed to have a mucolytic effect in traditional medicine books such as traditional chinese medicine and ayurveda, and it has been use hereditary in many countries. Previously has been described that the herbals combination containing *G.glabra*, *S.nigra*, and *Z.officinale* shows a potent mucolytic effect comparably to bromhexine (Rojas Urdaneta JE and Martínez-Sánchez,2010). In this study, we investigate the mucolytic effect from several natural herbal combination containing *G.glabra*, *S.nigra*, and *Z.officinale* with *E. purpurea* and *V. trifolia*. *E.purpurea* is a well known herbs that has immunomodulatory effect (Brinkernborn et al, 1999), while *V.trifolia* (Herdwiani,2011) is well known for its anti-inflammatoryand rhinitis alergy (Herdwiani,2011) . Therefore, those new combination is expected to bring a comprehensive cough treatment.

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MATERIALS AND METHOS

Experimental site

The ex-vivo study on mucolytic was performed in SOHO Center of Excellence in Herbal Research (SCEHR) Laboratory Jakarta Indonesia.

Materials

All reagents and solvents were purchased from commercial suppliers (Merck, JTBaker) and were used without further purification. Mucus was collected from fresh cleaned cow intestine. The testes natural herbal tablets were obtained from PT SOHO Industri Pharmasi. The composition of each tablet was derived from literature studies regarding its effective daily doses. Each tablet contains a combination of *E. purpurea* Herba Dry Extract 250 mg (Zhai et.al, 2017*), S.nigra* Fructus Extract 200 mg (Kong, 2009) , *G.glabra* Radix Extract 167 mg (EMA, 2013), *V.trifolia* Folium Extract 200 mg (Tandon, 2005), *Z.officinale* Rhizoma Extract 30 mg (Rouhi-Boroujeni et.al,2016).

A commercially available N-acetyl cysteine tablets were used as positive control. Viscosity analysis was measured using Viscometer Brookfield LVDV II.

Experimental

Phosphate Buffered Saline (PBS) pH 7.4 was freshly prepared by adding 8.0 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO into 800 mL demiwater. The pH was adjusted using HCl 6 M, and then demiwater was added to a total volume of 1 L.

Freshly collected mucus from a cleaned cow intestinal was diluted with PBS to get 20% m/v mucus-buffer solution and stirred to homogenized the mixture. Into 20% m/v mucus-buffer solution either NAC or NHC at various concentration was added. 0.1% w/v of NAC was used for positive control, and NHC concentrations of 0.25%, 0.5%, 1.0% and 2.0% were tested in the study. PBS without any treatment was used as blank and mucus in PBS without any treatment was used as negative control (Table 2).

After the addition of either blank, NAC or NHC treatment the mixture were stirred at 100 rpm for 5 minutes before all samples were then incubated at 37°C for 1 hour. After 1 hour incubation, viscosity of each solutioon was measure using Viscometer Brookfield LVDVII Spindel 61, and the measured viscosity was expressed in centipoise (cPs).

Statistical Analysis

The experiment were performed in triplicate. The deviation was stated as standard error of the mean (SEM*).* Statistic calculation using One-way ANOVA was performed using Minitab 17. Version 17.1.0. Statistical comparison was performed using the Tukey Pairwise Comparisons Method and 95% confidence.

RESULTS AND DISCUSSION

The mucolytic effect orserved through the reduction of mucus viscosity . NAC as expected show a strong mucoytic effect due to its ability to break down the sidulfide bond. Interestingly, NHC at all concentration showed a potent mucolytic effect and the aforementioned effect was also concentration dependent in which the effect become stonger with the increase of NHC concentration (Table 3).

ANOVA Single Factor analysis showed that all treatment groups (NHC at various concentration and NAC 0.1% w/v) are statistically significant difference ($p<0.05$) with PBS + mucus (negative control) group which indicate the mucolytic effect properties possesion from both NAC and NHC. Furthermore, the statistic analysis between NAC 1.0% w/v and various concentration NHC showed that almost all NHC treatment groups has no significant different efficacy in compare to the positive control, except the NHC I with 0.25% w/v. Neither inferiority nor superiority was observed between NAC and NHC, this indicates that in order to have similar mucolytic efficacy with 1.0% w/v N-acetyl cysteine, 0.5% w/v of NHC is required in cough treatment (Graphic

1). This result also suggest that the recommendation dose of NHC is around 2.95 – 4.40 g of herbal combination per day in order to have the similar efficacy to with NAC at its recommendation dose 400 – 600 mg per day. The mucolytic effect of NHC is more likely belongs to classic mucolytic class, since the ex-vivo experiment was conduct to a healthy mucus with DNA absence, thus DNA hydrolysis by dornase alfa is less likely to be occured.

The observed mucolytic effect from the NHC probably due to the synergistic mucolytic effect from glycyrrhizin, liquiritin apioside, liquiritin, and liquiritigenin from *G. glabra* (Liquorice) (Kuang et al., 2018), the antocyanidin rich - cyanidin-3-glucoside and cyanidin-3-sambubioside – *S. nigra* (elderberry) fructus extract (Bronum–Hansen and Hansen, 1983) and also 6-gingerol and 6-shogaol – a main constituent of *Z. officinale* (Pratap et al., 2017).

Taking all these data together we conclude that the natural herbal combination with *E. purpurea*, *S.nigra*, *G.glabra*, *V.trifoliae*, and *Z.officinale* has a great potency as an alternative mucolytic agent in cough medication. Although these study provide potency exploration of natural herbal combination, we realize further study regarding its mechanism of action could give more information and possible combination for a better treatment.

Mucolytic agents		**Possibly Mechanism of Actions**
Classical mucolytics	N-acetylcysteine	Severs disulfide bonds that link mucin oligomers. Anti-oxidant and antiinflammatory
	Nacystelyn	Increases chloride secretion and severs disulfide bonds
Peptide mucoytics	Dornase alfa	Hydrolyzes DNA polymer and reduces DNA length
	Thymosin β4	Depolymerizes filamentous actin

Table 1 Mucolytic agents

Solution	**Concentration**
Blank	PBS pH 7.4
Negative control	20% w/v mucus in PBS pH 7.4
Positive control	20% w/v mucus in PBS pH7.4 + 0.1% w/v N-Acetyl cysteine
NHC I	20% w/v mucus in PBS pH7.4 + 0.25% w/v NHC
NHC II	20% w/v mucus in PBS pH7.4 + 0.5% w/v NHC
NHC III	20% w/v mucus in PBS pH7.4 + 1.0% w/v NHC
NHC IV	20% w/v mucus in PBS pH7.4 + 2.0% w/v NHC

Table 2. Experimental condition at different concentration treatment agent

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No.	Treatment	I (cPs)	II (cPs)	III (cPs)	Average	SEM	p values
1	PBS (Blank)	0.24	0.36	0.30	0.30	0.03	
2	PBS +Mucus (Negative control)	8.04	8.10	8.04	8.06	0.02	
3	NAC 0.1% (Positive control)	2.94	3.08	3.36	3.13	0.12	*
4	NHC I - 0.25%	3.84	3.48	3.96	3.76	0.14	<0.05
5	NHC II - 0.5%	3.42	3.96	3.30	3.56	0.20	0.14
6	NHC III - 1.0%	3.36	3.30	3.36	3.34	0.02	0.16
7	NHC IV - 2.0%	3.42	3.66	3.00	3.36	0.19	0.35

Table 3. Sample solution viscosity

*p values was calculated using one-way ANOVA with Tuckey Pairwise Comparison Method to the positive control

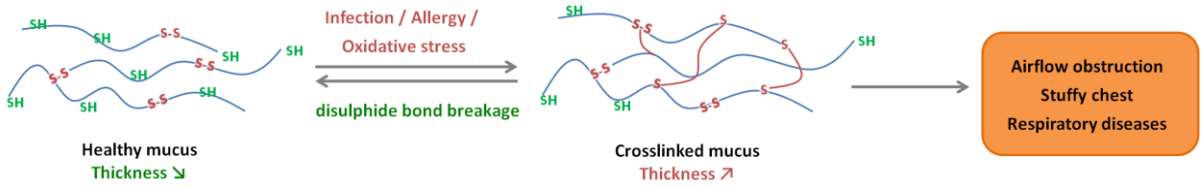


Figure 1 Classic mucolytic mechanism in breaking the disulfide bond to disentangle thecrosslinked mucus

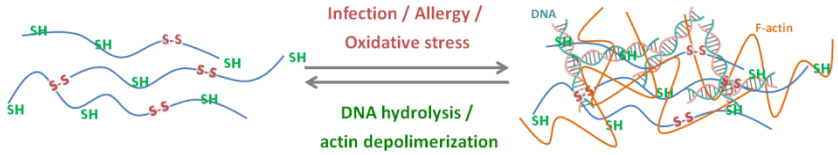
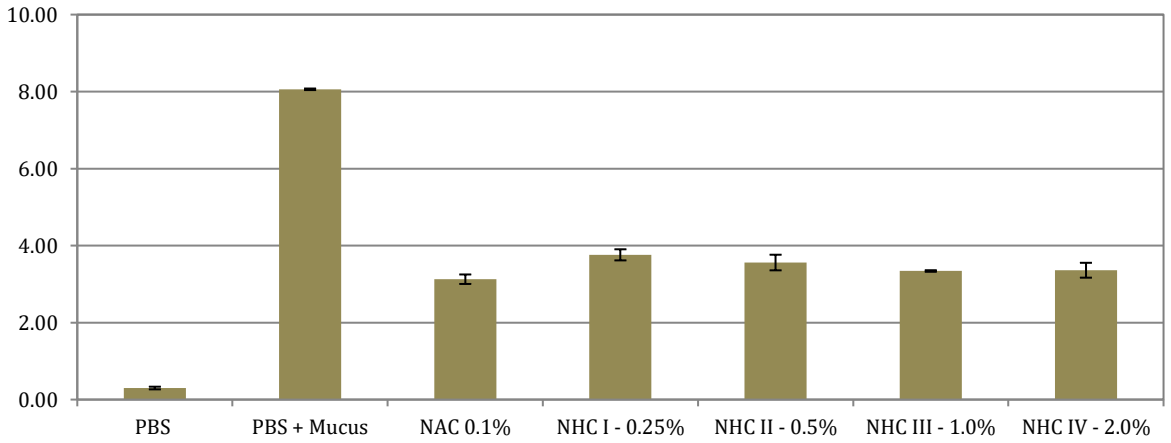


Figure 2 Peptide mucolytic mechanism through DNA hydrolysis by dornase alfa or via F-actin depolymerization



Graphic 1 Concentration dependent mucolytic activity in the presence of various concentrations of NHC, in its absence (PBS + Mucus) and in the presence of NAC as prositive control. The results were the average of three independent experiments with error bars (±SEM)

CONCLUSION

Natural herbal combination with *E.purpurea*, *S.nigra*, *G.glabra*, *V.trifolia*, and *Z.officinale* show interesting mucoactive activity. Addition of 0.5% of natural herbal combination reduce mucus viscosity to similar level with 0.1% NAC and the mucolytic effect was identified as dose-dependent. The use 0.5% of NHC was able to derived the recomendation dose of those combination, which are 2.95 – 4.40 g NHC per day by taking into account that the mucolytic effect is similar to 0.1% NAC and its recommendation dose is 400 – 600 mg per day. These finding suggest that the aforementioned natural herbal combination is a potent mucolytic agent for cough medication and interesting for further studies in term of its mucolytic mechanism and/or other therapeutic potency in cough medication.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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